PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTIFICATION OF ELECTION

(PCT Rule 61.2)

To:

Commissioner
US Department of Commerce
United States Patent and Trademark Office, PCT
2011 South Clark Place Room CP2/5C24
Arlington, VA 22202
ETATS-UNIS D'AMERIQUE

in its capacity as elected Office

Date of mailing (day/month/year) 04 May 2001 (04.05.01)	
International application No. PCT/NL00/00569	**Applicant's or agent's file reference** P50337PC00
International filing date (day/month/year) 14 August 2000 (14.08.00)	**Priority date** (day/month/year) 13 August 1999 (13.08.99)
Applicant DE GROOT, Ronald et al	

1. The designated Office is hereby notified of its election made:

   [X] in the demand filed with the International Preliminary Examining Authority on:

   12 March 2001 (12.03.01)

   [ ] in a notice effecting later election filed with the International Bureau on:

   _____

2. The election [X] was

   [ ] was not

   made before the expiration of 19 months from the priority date or, where Rule 32 applies, within the time limit under Rule 32.2(b).

The International Bureau of WIPO **34, chemin des Colombettes** **1211 Geneva 20, Switzerland**	Authorized officer Olivia TEFY
Facsimile No.: (41-22) 740.14.35	Telephone No.: (41-22) 338.83.38

'ATENT COOPERATION TREA'

PCT

INTERNATIONAL SEARCH REPORT

(PCT Article 18 and Rules 43 and 44)

Applicant's or agent's file reference	FOR FURTHER ACTION	see Notification of Transmittal of International Search Report (Form PCT/ISA/220) as well as, where applicable, item 5 below.
P50337PC00		
International application No.	International filing date *(day/month/year)*	(Earliest) Priority Date *(day/month/year)*
PCT/NL 00/00569	14/08/2000	13/08/1999
Applicant		
ERASMUS UNIVERSITEIT ROTTERDAM et al.		

This International Search Report has been prepared by this International Searching Authority and is transmitted to the applicant according to Article 18. A copy is being transmitted to the International Bureau.

This International Search Report consists of a total of 3 sheets.

[X] It is also accompanied by a copy of each prior art document cited in this report.

1. **Basis of the report**

   a. With regard to the **language,** the international search was carried out on the basis of the international application in the language in which it was filed, unless otherwise indicated under this item.

   [ ] the international search was carried out on the basis of a translation of the international application furnished to this Authority (Rule 23.1(b)).

   b. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international search was carried out on the basis of the sequence listing :

   [ ] contained in the international application in written form.

   [ ] filed together with the international application in computer readable form.

   [X] furnished subsequently to this Authority in written form.

   [X] furnished subsequently to this Authority in computer readble form.

   [X] the statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.

   [X] the statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished

2. [ ] **Certain claims were found unsearchable** (See Box I).

3. [ ] **Unity of invention is lacking** (see Box II).

4. With regard to the **title,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established by this Authority to read as follows:

5. With regard to the **abstract,**

   [X] the text is approved as submitted by the applicant.

   [ ] the text has been established, according to Rule 38.2(b), by this Authority as it appears in Box III. The applicant may, within one month from the date of mailing of this international search report, submit comments to this Authority.

6. The figure of the **drawings** to be published with the abstract is Figure No.

   [ ] as suggested by the applicant.

   [ ] because the applicant failed to suggest a figure.

   [ ] because this figure better characterizes the invention.

   [X] None of the figures.

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 00/00569

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/09 C07K14/315 C12N15/31 C07K16/12 A61P31/04
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

EPO-Internal, WPI Data, BIOSIS, PAJ, MEDLINE, CHEM ABS Data, EMBASE, LIFESCIENCES SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 18930 A (HUMAN GENOME SCIENCES) 7 May 1998 (1998-05-07) cited in the application *page 55, see sequence SP021* page 114 -page 116	1-21
A	WO 97 37026 A (SMITHKLINE BEECHAM) 9 October 1997 (1997-10-09) cited in the application page 346 -page 348	1-21

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 January 2001	05/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

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Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication,where appropriate, of the relevant passages	Relevant to claim No.
A	MCDANIEL L S ET AL: "COMPARISON OF THE PSPA SEQUENCE FROM STREPTOCOCCUS PNEUMONIAE EF5668 TO THE PREVIOUSLY IDENTIFIED PSPA SEQUENCE FROM STRAIN RX1 AND ABILITY OF PSPA FROM EF5668 TO ELICIT PROTECTION AGAINST PNEUMOCOCCI OF DIFFERENT CAPSULAR TYPES" INFECTION AND IMMUNITY,AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON,US, vol. 66, no. 10, October 1998 (1998-10), pages 4748-4754, XP000918186 ISSN: 0019-9567 the whole document	1-21
A	JANSEN W T M ET AL: "Use of highly encapsulated Streptococcus pneumoniae strains in a flow-cytometric assay for assessment of the phagocytic capacity of serotype-specific antibodies." CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, vol. 5, no. 5, 1998, pages 703-710, XP002158136 ISSN: 1071-412X the whole document	1-21
P,X	WO 00 06737 A (MICROBIAL TECHNICS LIMITED) 10 February 2000 (2000-02-10) cited in the application the whole document	1-21
P,X	OVERWEG K ET AL: "The putative proteinase maturation protein A of Streptococcus pneumoniae is a conserved surface protein with potential to elicit protective immune responses." INFECTION AND IMMUNITY, vol. 68, no. 7, July 2000 (2000-07), pages 4180-4188, XP002158137 ISSN: 0019-9567 the whole document	1-21

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INTERNATIONAL SEARCH REPORT

[redacted]nation on patent family members

International Application No
[redacted]T/NL 00/00569

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9818930 A	07-05-1998	AU 5194598 A	22-05-1998
		AU 6909098 A	22-05-1998
		EP 0942983 A	22-09-1999
		EP 0941335 A	15-09-1999
		WO 9818931 A	07-05-1998
		US 6159469 A	12-12-2000
WO 9737026 A	09-10-1997	EP 0907738 A	14-04-1999
		JP 2000511769 T	12-09-2000
WO 0006737 A	10-02-2000	NONE	

PATENT COOPERATION TREATY

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PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P50337PC00		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL00/00569	14/08/2000	13/08/1999
International Patent Classification (IPC) or national classification and IPC A61K39/09		
Applicant ERASMUS UNIVERSITEIT ROTTERDAM et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12/03/2001	23.10.2001
Name and mailing address of the international preliminary examining authority: European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Authorized officer Leber, T Telephone No. +49 89 2399 7195

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL00/00569

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-21 as originally filed

Claims, No.:

1-19 as received on 21/09/2001 with letter of 20/09/2001

Drawings, sheets:

1/2,2/2 as originally filed

Sequence listing part of the description, pages:

1-8, filed with the letter of 03.11.2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 1-19(partly);13,14,19(IA).

because:

☒ the said international application, or the said claims Nos. 13,14,19(IA) relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 1-19(partly).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-11,13-17,19(partly)
Inventive step (IS)	Yes:	Claims	
	No:	Claims	12,18(partly)
Industrial applicability (IA)	Yes:	Claims	1-12,15-18(partly)
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
se separate sheet

Re It m I
Basis of the opinion

1. Sequence listing pages 1-8 filed with the letter of 03.11.2000 do not form part of the application (Rule 13ter.1(f) PCT).

Re Item II
Priority

1. Priority of the present patent application was checked and found partly valid. The following sections of the description are not part of the priority document: page 2, lines 7-22; page 9, lines 20-27; page 10, line 30 - page 11, line 21; page 15, line 29 - page 17, line 18.

Re Item III
Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. Claims 13, 14 and 19 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

2. The sequence disclosed in Fig. 1b of the present application differs from the sequence provided in the sequence listing (Seq ID NO:2) by the first 9 amino acids. As the International Search Report is based on Seq ID NO:2, claims 1-19 have only partly been searched and examination will thus be restricted to searched parts (Rule 66.1(e) PCT).

Re Item V
Reasoned statem nt under Rule 66.2(a)(ii) with r gard to novelty, inventiv st p or industrial applicability; citations and explanations supporting such statem nt

1. Basis for the assessment of nov lty, inventiv st p and industrial applicability

1.1 Reference is made to the following documents:

D1: WO 98 18930 A (HUMAN GENOME SCIENCES) 7 May 1998 (1998-05-07) cited in the application

D2: WO 00 06737 A (MICROBIAL TECHNICS LIMITED) 10 February 2000 (2000-02-10) cited in the application

1.2 The amendments filed with the letter of 20.09.2001 fulfill the requirements of Art 34(2)(b) PCT.

2. Novelty

2.1 Document D1 discloses antigens and vaccines to prevent or attenuate infections caused by bacteria of the Streptococcus genus and S. pneumoniae in particular (D1, Abstract; page 115, claims 16 and 17). The vaccine encompasses a polypeptide or a fragment thereof contained in table 1 of D1 (D1, page 114, claim 16). Table 1 of D1 discloses SEQ ID 34, which is over a stretch of 141 amino acids (SKG...TEV) identical to that referred to in Fig. 1b of the present application. The vaccine may be prepared with a carrier and is suitable to elicit protective antibodies in the vaccinated animal (D1, page 114, claim 16). The peptides can be produced recombinantly (D1, page 3, line 36 - page 4, line 5) and encompass at least nine amino acids (D1, page 25, line 1). Moreover, the peptides may be used for antibody production (D1, claims 11 and 15). In light of the information provided in D1 it appears that D1 is fully enabling for the skilled person. Therefore claims 1-3, 8, 9, 11, 13-17, 19 lack novelty (Art 33(2) PCT).

This judgement is not altered by the fact that the name of the protein referred to in claim 1 ("protease maturation protein") is not disclosed in D1 as the name does not represent a distinguishing technical feature. Moreover, it is of no relevance that D1 does not disclose a opsonophagocytic response as this represents an intrinsic feature of the product referred to in claim 1, which is, as outlined above, not novel over D1. Thus, the product used in D1 has the same intrinsic feature of causing an opsonophagocytic response upon vaccination (see also ITEM V-3.1

below).

2.2 Claim 4 defines two strains from which the protease maturation protein can be derived. These strains do not provide a relevant feature for the assessment of novelty as the source of a product does not render the product novel. Thus claim 4 and also claims 5-7 lack novelty (Art 33(2) PCT).

2.3 Claim 10 represents product by process claim. Such claims are only allowable if each possible product is novel and inventive (Art 33(2) and Art 33(3) PCT). D1 discloses a segment of the polypeptide and its use for antibody production (see 2.1 above). In other words, antibodies are raised against the same target in D1 and in the present application. Therefore, claim 10 lacks novelty (Art 33(2) PCT).

3. Inventive step

3.1 Claim 12 differs from the closest prior art document D1 by the use of the protease maturation protein or a fragment thereof as a carrier. The term "carrier" is commonly understood as being a macromolecule suitable for enhancing the immunogenicity of the polypeptides. Examples are keyhole limpet hemacyanin (KLH), tetanus toxoid, pertussis toxin, bovine serum albumin and ovalbumin (e.g. D1, page 39, line 4-14). Thus, the function of the carrier appears to be to improve the epitope of the small peptide for the immune response of the challenged animal. It is therefore obvious for the person skilled in the art that in principal any protein could be used as a carrier. Moreover opsonisation represents a biological activity associated with mononuclear phagocytes and granulocytes which have the ability to ingest particulate matter. Both cell type mentioned above express cell surface receptors for various types of antibodies so that each matter which is bound to an antibody may be ingested by opsonisation once it is bound to an antibody. Therefore, activity of causing opsonophagocytosis appears not to be a no surprising effect of the carrier referred to in claim 12 but associated with any matter that may cause antibody production, e.g. the carriers mentioned above. In conclusion, claim 12 lacks an inventive step (Art 33(3) PCT).

3.2 Claim 18 refers to the use of the protease maturation protein or a fragment thereof for the preparation of a medicament for the treatment of diseases connected with

S. pneumonia infections. Claim 18 differs from the closest prior art documents D1 and D2 in that the medicament is for the treatment of diseases **connected** with S. pneumonia infections and not for the S. pneumonia infection as such.
The technical problem is thus an improved spectrum of applicability of the said medicament. An inventive step cannot be acknowledged (Art 33(3) PCT) for the solution of said problem as it is obvious for a person skilled in the art that a medicament which fights an infection is also of use for diseases which result from the said infection. As discussed above (see ITEM V-2.1) D1 appears to be enabling and is thus relevant prior art.

4. Industrial applicability

4.1 The subject-matter disclosed in the claims 8-10, 12, 15-17 of the present application appears to be industrially applicable (Art 33(4) PCT).

4.2 For the assessment of the present claims 1-7, 11, 13, 14, 18 and 19 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII
Certain observations on the international application

1. Claim 1 lacks clarity (Art 6 PCT). The name "protease maturation protein" is insufficient to define the protein concerned. Further, the said protein "comprises" the amino acid sequence as shown in Fig. 1B. It is thus not clear whether or not the said amino acid sequence defines already a protease maturation protein (Art 6 PCT). Moreover, the terms "fragment", "homologous", "functional homologous", "functional fragment" lack clarity (Art 6 PCT) as there is no clear definition how, for example, a fragment has to look like to be still a "protease maturation" and to still

have the relevant function. No clarification can be derived from the description for these terms. The term "fragment" is on the one side defined functionally (page 7, line 17) and on the other side in terms of the peptide length (page 8, lines 6-7) without linking these definitions so as to render it clear whether or not, for example, the required biological activity is present. Moreover, the function of Pmp appears to be insufficiently disclosed as it is only based on sequence homology analysis (page 6, lines 24-29).
At least some of the said objections apply also to claims 5, 6, 8, 9, 11, 12, 15-19.

2. Claims must not in respect of technical features rely on references to drawings (Rule 6.2a PCT). Amino acid sequences may be defined with sequence identification numbers. This objection applies to claims 1, 8, 9, 11, 12, 15-19.

3. The term "suitable" in claims 3 and 14 lacks clarity as no definition is given which permits the skilled person to distinguish between suitable and unsuitable carriers (Art 6 PCT).

4. The terms "anchoring fragment", "antigenic fragment or functional equivalent thereof" and "functional equivalent of a receptor binding site or a antibody binding site" in claim 6 lack clarity (Art 6 PCT).

5. Considering the nature of the invention, it appears that the number of 14 independent claims is excessive leading to a lack of conciseness (Guidelines, Section IV, III-5).

6. Claim 18 refers to the use of the protease maturation protein or a fragment thereof for the preparation of a medicament for the treatment of diseased **connected** with S. pneumonia infections. This feature appears not to be supported by the description (Art 6 PCT).

REPLACED BY ART 34 AMDT

Claims

1. A vaccine or medical preparation comprising a protease maturation protein of *S. pneumoniae* and/or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof for the treatment of microbial infections.
2. The vaccine or medical preparation according to claim 1 for the treatment of *S. pneumoniae*.
3. The vaccine or medical preparation according to claim 1 or 2, further comprising a suitable adjuvant or carrier.
4. The vaccine or medical preparation according to anyone of the claims 1-3, wherein said protein comprises an amino acid sequence as shown in fig 1B.
5. The vaccine or medical according to anyone of the claims 1-4, wherein said protein is the protein maturation protein from *S. pneumoniae* Ft231 or EF3296.
6. The vaccine or medical preparation according to anyone of the claims 1-5, wherein said fragment comprises an anchoring fragment, an antigenic fragment or a functional equivalent thereof or a functional equivalent of a receptor binding site or an antibody binding site.
7. The vaccine or medical preparation according to anyone of the claims 1-6, wherein said protein or said fragment comprises a purified, partly purified, recombinant or synthetic protein or fragment thereof.
8. The vaccine or medical preparation according to anyone of the claims 1-7, wherein said fragment comprises at least 8 amino acids.
9. Method for the preparation of a vaccine against *S. pneumoniae* comprising the steps of:

   a. isolating a protease maturation protein of *S. pneumoniae* or a fragment thereof or a recombinant or synthetic protein or fragment thereof or homologous or functionally homologous protein or fragment thereof; and

   b. combining the protein or the fragment thereof obtained under (a) with a suitable carrier or adjuvant.
10. Method for obtaining an antibody against the protease maturation protein of *S. pneumoniae*, comprising the steps of isolating said protease maturation protein

or a fragment thereof, and raising antibodies against said protein or fragment thereof.

11. Antibody obtainable by the method according to claim 10.

12. Use of a protease maturation protein of *S. pneumoniae* or a fragment thereof for the preparation of a vaccine for the treatment or prophylaxis of a *S. pneumoniae* infection.

13. Use of a protease maturation protein of *S. pneumoniae* or a fragment thereof or a recombinant or synthetic protein or fragment thereof as a carrier.

14. Method of treatment of a *S. pneumoniae* infection comprising administering a vaccine according to claims 1-8.

15. Method for the vaccination of a mammal against an infection of *S. pneumoniae* comprising administering a suitable dose of a vaccine according to anyone of the claims 1-8.

16. Use of a nucleic acid sequence encoding for a protease maturation protein or a fragment thereof for obtaining a recombinant protease maturation protein or fragment thereof.

17. Cell containing a recombinant nucleic acid sequence or a vector encoding for protease maturation protein or a fragment thereof.

18. Recombinant protease maturation protein or fragment thereof obtainable through the expression of a gene sequence encoding for said protein in a suitable vector.

19. Use of protease maturation protein of *S. pneumoniae* and/or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof for the preparation of a medicament for the treatment of diseases connected with *S. pneumoniae* infections.

20. Use of protease maturation protein of *S. pneumoniae* and/or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof for eliciting opsonophagocytic activity and/or *in vivo* immunisation and/or *in vivo* immune protection against *S. pneumoniae*.

21. Method for the identification of proteins eliciting opsonophagocytic activity and/or *in vivo* immune protection comprising subjecting proteins to protein electrophoresis, preferably 2D, obtaining antisera against the surface associated

proteins, subjecting the isolated protein or fractions thereof to an immunocytometric assay and to an opsonophagocytic assay, in any order.

Title: Pneumococcal vaccines.

The invention relates to the field of vaccines against microbial infections and especially bacterial vaccines, in particular to pneumococcal vaccines.

Streptococcus pneumoniae (pneumococcus, *S. pneumoniae*) is an important pathogen, which causes significant morbidity and mortality throughout the world. *S. pneumoniae* is a major cause of invasive diseases such as meningitis, bacteremia, and pneumonia, as well as non-invasive diseases like acute otitis media and sinusitis (1). In young children, the pneumococcus is often part of the normal nasopharyngeal flora. Especially during the first two years of life, children are colonised with novel strains of pneumococci. Children colonised with *S. pneumoniae* develop more often acute otitis media than children who are not colonised (2, 3, 4).

The precise molecular mechanisms through which the pneumococcus invades and damages host tissues are not fully understood. For many years, the polysaccharide capsule has been recognised in the art as the major virulence factor and, consequently, an important vaccine candidate (for review, see 5, 6). The current pneumococcal vaccine strategies focus on the use of conjugates, in which a limited number of different capsular polysaccharides are linked to a carrier protein (7,8). Although the results of early trials look promising, problems still arise since large-scale vaccination over time generally leads to a shift in serotype distribution towards capsular types that are poorly immunogenic or not included in the vaccine. Such a shift may be enhanced by the frequent horizontal exchange of capsular genes, as described by several investigators (9, 10, 11).

Over the last few years, much attention has been focused on the role of pneumococcal proteins in pathogenesis and protection. Proteins that are involved in the pathogenesis of infections by *S. pneumoniae* are considered to be interesting components for future conjugate vaccines. Such proteins are able to switch the immune response against the polysaccharides present in the vaccine from T-cell independent to T-cell dependent, through which the antibody response towards the polysaccharides may be increased and a memory response will be provided. In addition, such proteins should provide protection against colonisation and infection

with *S. pneumoniae* strains whose capsular polysaccharides are not included in the vaccine.

The protective abilities of various (virulence) proteins have been investigated previously. Immunisation of pneumolysin (12), pneumococcal surface protein A (PspA) (13, 14,15) pneumococcal surface adhesin A (PsaA) (16), and neuraminidase (17) clearly confer protection in animals.

In the literature various polynucleotides of *S. pneumoniae* and polypeptides predicted to be encoded by said nucleotides have been reported and the use of these compounds in vaccines and medicinal preparation has been contemplated, for instance in WO 97/37026 and WO 98/18930. These publications however, do not identify any functional protein let alone a vaccine based on a functional protein. These publications are further silent in respect of proteins that when used in vaccines are able to elicit an immuneresponse let alone that they are able to elicit any protective, more in particular opsonophagocytic activity.

The publications further do not disclose any information regarding cross reactivity towards various strains of *S. pneumoniae* in a relevant vertebrate host. Furthermore these publications do not describe the protease maturation protein of *S. pneumoniae*. Another publication that relates to the present invention is WO 00/06737. This publication discloses a pool of several hundreds of proteins. Most of these proteins, including the protein described in the present invention have not been tested for their immuneresponsive properties, opsonophagocytic activity or cross reactivity.

The present invention identifies surface-associated proteins from *S. pneumoniae* with immune-protective properties, more in particular opsonophagocytic activity. Furthermore the present invention provides the use of these proteins as vaccine components and their use in conjugate vaccination strategies. The invention further provides for antibodies which express opsonophagocytic activity and methods for their production, as for example detailed in the experimental part.

It has now been found that a surface-associated protein of *S. pneumoniae* can be used in the preparation of a vaccine against micro-organisms and especially *S. pneumoniae*. This surface protein is present in a large number of strains of *S. pneumoniae*.

The invention accordingly relates to a vaccine or medical preparation comprising a protease maturation protein of S. pneumoniae and/or a fragment thereof and/or a homologous and/or functionally homologous protein and/or fragment thereof for the treatment of microbial infections and especially of *S. pneumoniae* infections and for the generation of antibodies in an immunised or vaccinated in a vertebrate host and which expresses opsonophagocytic activity against *S. pneumoniae* and infections thereof. The invention also relates to the use of protease maturation protein of *S. pneumoniae* or a fragment thereof for the preparation of a vaccine for the treatment of a *S. pneumoniae* infection and/or colonisation and to the use of a protease maturation protein of *S. pneumoniae* or a fragment thereof or a recombinant or synthetic protein or fragment or functionally homologous protein or fragment thereof as a carrier for inducing prophylactic protection against other micro-organisms including viruses.

In this description and the appending claims treatment encompasses and generally is the prophylaxis of infections.

Surface-associated proteins were isolated or purified from the *S. pneumoniae* strains FT231 and EF3296, respectively, using either the SB14 extraction procedure or the Triton X114 extraction procedure as further illustrated in the working examples herein-below. The proteins and polypeptides were purified in relatively high concentrations, as shown by two-dimensional SDS-PAGE. Extracts from either strain resulted in a highly homologous protein profile as demonstrated by computer-assisted analysis. Since both extraction procedures resulted in comparable protein profiles, the SB14 extraction procedure was used for further experiments.

Hyperimmune serum antibodies were raised against the pneumococcal surface-associated proteins of *S. pneumoniae* strains FT231 and EF3296, respectively. To confirm the presence of surface-exposed proteins in the fraction, the sera were tested for the recognition of components at the surface of pneumococcal whole cells. Immuno-cytometric experiments demonstrated the recognition of components exposed at the surface of the homologous pneumococcal strains by the hyperimmune sera. Heterologous immuno-cytometric analysis demonstrated that the serum-recognition of components at the surface of the two strains display partial overlap as the level of fluorescence of the bacteria using the homologous serum was greater than the fluorescence level using the heterologous serum. In addition,

components at the surface of eleven other pneumococcal strains, which display ten distinct genotypes and represent eight clinically important serotypes, were invariably recognised by the hyperimmune sera. The strains which have tested are described in more epidemiological detail by Hermans et al. (10).

Hyperimmune rabbit sera raised against the surface-associated pneumococcal proteins in the phagocytosis assay as described by Alonso Develasco et al. (5) have been analysed. The *in-vitro* opsonophagocytic activity of the serum is presumed to correlate with *in-vivo* protection against *S. pneumoniae*. The opsonophagocytic activity of the hyperimmune sera was high using the homologous pneumococcal strains. The specificity of the serum opsonophagocytic activity was determined using seven genotypically distinct pneumococcal strains, representing seven serotypes that cause most infections in young children and two strains of the genetically closely related species *S. bovis* and *Enterococcus faecalis*, respectively. The hyperimmune rabbit sera were invariably opsonically active against the pneumococcal strains. In contrast, the serum opsonophagocytic activity was very low using *S. bovis* and *E. faecalis*. This means that *S. bovis* and *E. faecalis* are not recognised by the serum. Apparently these organisms have insufficient homology to *S. pneumoniae* for serological recognition.

All immunodominant proteins were cut from two-dimensional acrylamide gels. Protein characterisation was performed using mass spectrometric analysis (Maldi-tof) to analyse trypsine fragments on the amino acid level. In addition, monospecific hyperimmune rabbit serum antibodies were raised against the acrylamide-embedded proteins. The monospecific hyper-immune sera were used to identify the cellular localisation of the proteins by immuno-electron microscopy and to determine the capacity of these proteins to elicit opsono-phagocytosis.

Blast and/or Blastp computer programs were used for comparison of the sequence of the protein isolated from *S. pneumoniae* with known sequences in various databases. In this program the Expect value (E-value) is a parameter that describes the number of hits that can be expected just by chance when searching a database. The E value is a measurement for the random background noise that exists from a match between two sequences. To decide whether or nbot a protein is functionally homologous with Pmp, a homology cut-off value is defined as an E-value of 10^{-10}. A

protein with an E-value of more than 10^{-10} is not considered sufficient homologous to Pmp from *S. pneumoniae*.

One of the proteins revealed to be homologous to a polypeptide encoded by nucleotide sequence 7632-8597 on contig 33 of *S. pneumoniae* (Figure 1). This ORF was identical to ORF 414 of *S. pneumoniae* in the WIT-system. Details about the WIT system can be found on http://wit.mcs.anl.gov/ and on the website of The Institute for Genomic Research, Rockville USA updated on April 7, 1999.

Since this pneumococcal polypeptide was related to protease maturation protein *Lactobacillus paracasei* (Swiss Prot acc. nr. Q02473) (Figure 2), and *Lactococcus lactis subspec. lactis* (Swiss Prot acc. nr. P15294) (Figure 3) and *Lactococcus lactis subsp. cremoris* (Swiss Prot acc. nr. P14308) (Figure 4) it was designated the protease maturation protein (Pmp) of *S. pneumoniae*. Also the molecular weight of the protein cut from the acrylamide gel corresponds with the molecular weight of Pmp.

This protein has various interesting characteristics with respect to its use in conjugate vaccines.

The immuno-electron microscopy using the monospecific rabbit antibodies raised against Pmp demonstrated that this protein was surface-associated.

The opsonophagocytic activity of the monospecific anti-Pmp rabbit antibodies was measured using the homologous pneumococcal strain, as well as seven genotypically distinct pneumococcal strains, representing seven serotypes that causes most infections in young children and two strains of the genetically closely related species *S. bovis* and *E. faecalis*, respectively. The anti-Pmp rabbit antibodies were invariably opsonically active against the pneumococcal strains. In contrast, the serum opsonophagocytic activity was very low using *S. bovis* and *E. faecalis*. These data show that Pmp has the ability to elicit immune protection, which is a major requisite with respect to its use as a vaccine component. Thus, not only the existence of the protein has been demonstrated, also its potential function and properties have been adequately established, which distinguishes the present invention over the art.

DNA sequence analysis of the *Pmp* genes of the homologous pneumococcal strain, as well as fifteen genotypically distinct pneumococcal strains, representing fourteen serotypes that cause most infections in young children demonstrated very limited variation. This is an important feature of Pmp with respect to its use as a

vaccine component, and of the present invention in general, as it will guarantee immunological cross reactivity.

Phenotypic variation is an important mechanism allowing bacterial pathogens to adapt to different host environments. In *S. pneumoniae,* phenotypic variation due to alterations in cell-surface structures can be detected as spontaneous, reversible changes in colony morphology. Such alterations result in opaque and transparent colonies within single strains. The relationship of several previously identified cell-surface structures to phenotypic variation has recently been described (18). The transparent phenotype incorporates significantly more surface-exposed phosphorylcholine. In addition, the expression of three choline-binding proteins (Cbp) also varies in the phenotypic variants. The expression of autolysin LytA, is lower in opaque variants as compared to transparent variants, pneumococcal surface protein PspA is present in higher amounts in opaque variants, and CbpA is present in higher amounts in transparent variants. Such phenotypic changes also result in alterations in virulence phenotype. The opaque phenotype has decreased ability to colonise the nasopharynx as compared to the transparent phenotype (19). In addition, the survival time of mice after intraperitoneal challenge of the opaque phenotype is decreased as compared to the transparent phenotype (20)

Pmp is predominantly present in transparent colony variants of *S. pneumoniae.* Since these variants are prone to colonise the nasopharynx in animal models (21), immunisation with conjugate vaccines containing Pmp or Pmp components will enhance the removal of colonising pneumococci from the nasopharynx.

The determination of the function of Pmp in *S. pneumoniae* has been based on the homology of the protein with Pmp proteins of other bacterial species. The function of the Pmp proteins of other bacterial species is generally the activation of certain proteases. The most important keys to the use of Pmp in vaccines is the surface exposure of Pmp, whereby Pmp is available to the immunesystem and the elicitation of opsonophagocytic activity as shown in the opsonophagocytosis assay.

Pmp has been identified herein as a conserved protein. This means that Pmp is expressed in many, if not all strains of *S. pneumoniae.* Pmp has been shown to have surface exposure and to elicit opsonophagocytic activity. These characteristics of Pmp enable the use of this protein and protein fragments or functional equivalents

thereof in the preparation of the vaccine in such manner that the vaccine can be used against nearly all strains of *S. pneumoniae*. As Pmp is depicted as the protease maturation protein *of S. pneumoniae* and as this protein has a function as a protease activator, it is therefor easily envisaged that the protease activator proteins of other bacterial species, especially of the genus *Streptococcus*, will fulfil a major role in pathogenesis. Similar protease activators from other species, for instance from *Meisseria*, can likewise be used in vaccine preparations. These homologues and functional homologues of Pmp can thus be used in the preparation of a vaccine for other bacterial species than *S. pneumoniae*. The present invention therefor also encompasses the homologues and functional homologue equivalent proteins of Pmp and fragments and their use in vaccine preparations.

In a preferred embodiment of the invention the protein or fragments thereof used in the preparation of the vaccine, is the Pmp or a (functional) homologous fragment thereof of *S. pneumoniae* strain FT231 or strain EF 3296.

It is likewise possible to employ a fragment of Pmp for the preparation of a vaccine. A fragment is a polypeptide with an amino acid sequence which is functionally similar to the corresponding section of the protein. In principle any fragment of Pmp can be used. A preferred fragment is an oligopeptide that contains one of the characterising parts or active domains of the protein. The fragment of Pmp can be (part of) an anchoring fragment, an antigenic fragment or a fragment that is (part of) a receptor binding site or an antibody binding site or combinations thereof. The Pmp or the fragment or the functional equivalent thereof can be obtained by recombinant techniques or by chemical synthesis of Pmp oligopeptides. Synthetic oligopeptides based on or derived from Pmp can for instance be obtained by conventional pepscan technology. The use of Pmp or a (homologous) fragment or a (homologous) functional equivalent thereof as a carrier in other vaccines is also encompassed by the invention. When Pmp expresses certain strongly immunogenic properties, these properties can be used by employing Pmp as a carrier. Pmp then serves to induce an immune response to a bad immunogen such as a protein or a sugar of other bacterial or viral pathogens. This strategy is useful in conjugate vaccine strategies. In an embodiment the protease maturation protein or (homologous) fragment or (homologous) functional equivalent thereof is used as a carrier protein, preferably in a conjugate vaccine strategy.

In a preferred embodiment of the invention, the fragment is an anchoring fragment, an antigenic fragment or a functional equivalent fragment thereof or a functional equivalent for a receptor binding site or an antibody binding site.

A fragment of Pmp in general will consists of an oligopeptide of at least 5 amino acids, preferably at least about 8, but oligopeptides with 10-15 amino acids are preferred. These fragments can also be used in the from of tandem oligopeptides or dimerised oligopeptides.

The protein or (functional) fragment that is used in the preparation of the vaccine can be a partially purified protein, a purified protein or fragment of Pmp.

In order to obtain a vaccine that can be administered, the protein is brought into a form that is suitable for this purpose. To this end, the protein can be conjugated with a carrier protein. Carrier proteins that can be used in this invention are in general conventional carriers and as such are well known in the art. The vaccine can likewise also comprise adjuvants and other additional components to further ensure the proper functioning of the vaccine. These additional components are generally known by the skilled man.

In a preferred embodiment of the invention, the composition comprising the protein or the fragment is therefore combined with an adjuvant and/or a carrier. From this composition a vaccine is prepared which is used in the preventive vaccination against *S. pneumoniae*. A more preferred embodiment of the invention comprises protease maturation protein of *S. pneumoniae* or a fragment thereof for the preparation of a vaccine for the preventive treatment of a *S. pneumoniae* infection.

The invention further provides for a method for the preparation of a vaccine against S. pneumoniae. The method comprises the steps of preparing or isolating the protein or the fragment or homologue or functional homologue of the protein or fragment, determining the immunogenic response by raising antibodies against the protein or the fragment or homologue or functional homologue of the protein or fragment and testing the antibodies for activity. The method according to the invention also encompasses the recombinant or synthetic production of the protein or the fragment or homologue or functional homologue of the protein or fragment and the subsequent steps to the preparation of the vaccine.

In general, in this invention, when a protein or a fragment thereof is described, the protein and the fragment encompass the Pmp of *S. pneumoniae* or a

fragment thereof, a homologous protein or fragment or a homologous or functional homologous protein or fragment thereof.

A preferred embodiment of the invention is a method for the preparation of a vaccine against *S. pneumoniae* comprising the steps of :

a. obtaining a protease maturation protein of *S. pneumoniae* or a fragment thereof or homologous or functionally homologous protein or fragment thereof; and

b. combining the protein or the fragment obtained under (a) with a suitable carrier or adjuvant.

The invention further provides a method for the vaccination of a mammal against an infection of *S. pneumoniae* comprising administering a suitable dose of the vaccine of the invention. The vaccine is suitable for vaccination against all strains and subspecies of *S. pneumoniae,* also for veterinary purposes.

The invention provides for the use of homologous Pmp proteins or fragments thereof of other *S. pneumoniae* species with amino acid sequences or fragments thereof such as peptides that are functionally homologous to the sequence depicted in fig 1B. Said functional homologous peptides can be used in a vaccine for the treatment, preferably the preventive treatment of a wide variety of strains and (sub)species of *S. pneumoniae.*

In one aspect of the invention the antibodies raised against the protein of the present invention may also provide for neutralising effects. These antibodies do not raise any opsonophagocytic activity against *S. pneumoniae* or only to a reduced extent These antibodies merely block certain epitopes of the antigen (in this case Pmp) and may disturb secretion, protection or activation of proteins, directly or indirectly involved in pneumococcal pathogenesis aspects, including colonisation and other processes of *S. pneumoniae.* This provides for an alternative way of treating *S. pneumoniae* infections.

The sequence of the *S. pneumoniae* nucleotides 820800-821738 on contig 3836 (previously known as 7632-8597 on contig 33) and the encoding polypeptide sequence harbouring Pmp are known. The nucleic acid sequence can be used to encode for Pmp or a fragment thereof. By incorporating this sequence or part thereof in a suitable vector and expressing that vector in a cell, it is possible and within the scope of the

invention to obtain recombinant peptide sequences which can subsequently be used in the preparation of a vaccine.

Accordingly the invention also relates to the use of the nucleic acid sequence or fragment thereof or a (functionally) homologous sequence or fragment thereof encoding for Pmp or a fragment thereof. The invention also provides a method for the preparation of a vaccine against *S. pneumoniae*. The method comprises the principal steps of isolating the Pmp protein or the fragment thereof, determining the immunogenic response by raising antibodies against the protein or the fragment, and testing the antibodies in *S. pneumoniae* strains. The invention also provides the recombinant protein or fragment thereof, that has been obtained, for instance, through the expression of a gene sequence encoding for the protein in a suitable vector. The invention also provides for a method of obtaining an antibody and to the antibody. An embodiment of the invention is therefor a method for obtaining an antibody against protease maturation protein comprising the steps of isolating a protease maturation protein or a fragment thereof, raising antibodies against the protein or fragment thereof and isolating the antibodies. The protein or fragment that is used in the preparation of the vaccine or in obtaining the antibody can be a recombinant or synthetic protein or fragment of Pmp.

In an embodiment of the invention, the vaccine can also be derived from the expression of recombinant nucleic acids. The Pmp gene of *S. pneumoniae* can suitably be expressed in *E. coli*.

Pmp and derivatives such as fragments for instance in the form of oligopeptides and modified oligopeptides are tested in animal models to elicit the protection against the different forms of infection (otitis media, pneumonia, sepsis, meningitis) and colonisation.

The production of Pmp for vaccine purposes is in a recombinant form wherein the gene encoding for Pmp is overexpressed in gram positive and/or gram negative bacteria. This yields Pmp in bulk quantities after which further necessary steps such as purification follow.

The present invention further pertains to a method for the identification of proteins expressing opsonophagocytic activity comprising extraction of, preferably surface associated, proteins, subjecting the obtained proteins to protein

electrophoresis, preferably 2D, obtaining antisera against the proteins, and subjecting the antibodies to an opsonophagocytic assay.

The method provides for a rapid and efficient screening of a large number of proteins or fragments thereof and allows for the rapid identification of proteins of interest. The method according to this aspect of the invention is surprising in that the combination protein electrophoresis and an opsonophagocytic assay results in proteins that are considered to have immunoprotective properties. Electrophoresis techniques use denatured proteins. Antibodies that are active in opsonophagocytic assays are preferably directed to epitopes of the native protein. It is a surprising aspect of the present invention that by the combination of these two methods, antibodies are obtained that allow for immunoprotective properties.

Alternatives for the opsonophagocytic assay are *in vivo* passive immunoprotection assay, *in vivo* active immunoprotective assay and *in vivo* active immunoprotective assay. These techniques are by itself well known in the art and may also serve to identify vaccine candidates according to the invention. By varying the extraction techniques, for instance by varying the detergent or by using chromatographic techniques such as column chromatography, protein fractions of varying composition can be isolated which can be further processed according to the method. It is likewise possible to directly identify the proteins after the electrophoresis step, prior to assaying the proteins for instance by using mass-spectroscopic techniques such as Maldi-tof.

Description of the Figures:

Figure 1 : the *S. pneumoniae* nucleotides 820800-821738 on contig 3836 (http://www.tigr.org/data/*S.pneumoniae* /) (A) and the encoding polypeptide sequence (B) harbouring Pmp. The presumed methionine start codon of Pmp is depicted in bold and underscored.

Figure 2 : The protease maturation protein of *Lactobacillus paracasei* (Swiss Prot acc. nr. Q02473).

Figure 3 : The protease maturation protein of *Lactococcus lactis subspec. lactis* (Swiss Prot acc. nr. P15294)

Figure 4 : The protease maturation protein of *Lactococcus lactis subsp. cremoris* (Swiss Prot acc. nr. P14308)

MATERIALS AND METHODS

Extraction of surface-associated, hydrophobic proteins of *S. pneumoniae.*

S. pneumoniae FT231 and *S. pneumoniae* EF3296 were cultured at 37 °C in Todd Hewitt broth (Difco laboratories, Detroit, USA) supplemented with 0.5% Yeast Extract (Difco laboratories). At logarithmic growth phase (OD_{550}=0.3) the bacteria were harvested by centrifugation, and washed three times with phosphate-buffered saline pH 7.5 (PBS). After the final washing the bacteria were resuspended in TE-buffer (10 mM Tris-Cl, 1 mM EDTA). The cells were disrupted by ultrasonic treatment (Branson sonifier 250, Branson Ultrasonics, Danburry, USA).

Extraction with sulfobetaïne 14 (SB14) was performed as described by Schouls *et al.* (22). In brief, the water-soluble cytoplasmic proteins were removed by washing the bacterial lysates five times with PBS. Cell walls, membranes and other particulate material were collected by centrifugation at 48,400*g for 20 min. Pellets were resuspended in 150 mM NaCl and centrifuged for 20 min at 48,400*g. The pellets were then incubated for 2 hours at room temperature with 0.25% N-tetradecyl-N,N-dimetylammonio-1-propanesulfonate (SB14, Serva, Heidelberg, Germany) in the presence of 150 mM NaCl, 10 mM $MgCl_2$ and 10 mM Tris-HCl pH 8.0 during constant stirring. The hydrophobic, membrane-associated proteins were recovered as described by Wessel and Flügge (23).

Extraction with Triton X114 (Sigma, St. Louis, USA) was also performed as described by Schouls *et al.* (24). Briefly, bacterial lysates were centrifuged at 20,000*g for 20 min. Pellets were dissolved with 1% Triton X114 in PBS for 1 hour at 0 °C.

After extraction, the suspensions were centrifuged at 25,000* g at 4 °C for 1 hour, the supernatants were incubated at 37 °C for 30 min, and centrifuged at 25,000*g at 25 °C for 1 hour to separate the detergent phase and aqueous phase. The proteins in the detergent phase were extracted according to the procedure of Wessel and Flügge (23). Protein concentrations were measured by the method of Bradford (25).

Protein electrophoresis and staining.

One-dimensional sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was carried out in the Biorad minigel system with 13% polyacrylamide gels. The samples were dissolved in sample buffer (10 mM Tris-HCl, 1 mM EDTA, 1% SDS, 10 mM DTT, 1% glycerol, 0.01% bromophenol blue indicator (Merck, Darmstadt, Germany), boiled for 5 min and subjected to electrophoresis (26).

Two-dimensional SDS-PAGE was performed according to the instructions of the manufacturer (Pharmacia Biotech, Uppsala, Sweden) including modifications of Rabilloud *et al.* (27). After isoelectric focusing, proteins were separated using gradient (12-20%) polyacrylamide gel electrophoresis.

Silver staining of polyacrylamide gels was performed as described by Blum *et al.* (28). In addition, standard procedures were used to stain the polyacrylamide gels using Coomassie brilliant blue (CBB) (26).

The software program PD Quest (PDI, New York, USA) was used for the computerised analysis of two-dimensional SDS-PAGE gels.

Hyperimmune rabbit antiserum.

Hyperimmune antiserum was raised against the hydrophobic, surface-associated proteins by injecting New-Zealand White rabbits subcutaneously into 4-5 places. The SB14 and Triton X114-extracted hydrophobic surface-associated proteins (500 µg) of *S. pneumoniae* FT231 and EF3296, respectively, were dissolved in 0.5 ml 0.9% NaCl, and subsequently mixed with 0.5 ml Freund's incomplete adjuvant (Pierce, Rockford, USA). In addition, hyperimmune rabbit serum was raised against SB14-purified hydrophobic surface-associated proteins of *S. pneumoniae* FT231 that

were subjected to 1D-SDS-PAGE. The total protein pool was cut from the polyacrylamide gel, washed three times with 0.1 M NaAc, 96% EtOH, ground into a fine suspension in 0.5 ml PBS, and subsequently mixed with 0.5 ml Freund's incomplete adjuvant. Negative control serum was gained by injection of washed and ground polyacrylamide in 0.5 ml PBS mixed with 0.5 ml Freund's incomplete adjuvant. The primary injection was followed by four subcutaneous booster injections at four-week intervals.

Antibodies to type 2 capsule were purchased from Statens Seruminstitut, Copenhagen, Denmark. Recombinant pneumolysin was used to raise hyperimmune sera in rabbits as described previously (29). These sera were used as positive controls in passive immunisation experiments.

Indirect immuno-cytometric assay.

Pneumococci were grown to logarithmic phase in Todd-Hewitt broth supplemented with 0.5% Yeast Extract at 37 °C using 5% CO_2, then washed three times in ice-cold PBS and stored overnight at 4 °C. The bacteria were incubated in 5% rabbit serum (10^7 bacteria in 20 µl final volume) for 15 min at 4 °C while shaking. The bacteria were washed twice using ice-cold PBS and incubated for 15 min at 4 °C with 20 µl (1:5 dilution) of fluorescein-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, USA) while shaking. The bacteria were washed twice with ice-cold PBS and resuspended in 100 µl of ice-cold paraformaldehyde (0.5 %) in PBS. The samples were analysed in a FACScan flow cytometer (Becton Dickinson, Mountain View, USA).

Phagocytosis assay.

Analysis of the opsonophagocytic activity of the sera was performed as described by Alonso Develasco *et al.* (30). In brief, *S. pneumoniae* was grown to logarithmic phase in Todd-Hewitt broth supplemented with 0.5% Yeast Extract at 37 °C using 5% CO_2. After washing with PBS, the bacteria were labeled with fluorescein-isothiocyanate (FITC, isomer I, Sigma Chemical Co., St. Louis, USA) (0.5 mg/ml in

PBS) for 1 hour at 4 °C, washed twice and resuspended in Hank's balanced salt solution (HBSS) containing 1% w/v bovine BSA. The bacteria (10^8 bacteria per 100 µl BSA-HBSS) were stored at
-20 °C. Samples of 2.5 * 10^6 bacteria were transferred into round-bottom microtiter plates (Greiner Labortechnik, Alphen a/d Rijn, The Netherlands). Rabbit sera diluted in BSA-HBSS and heat-inactivated for 30 min at 56 °C were added to the bacteria (final volume 50 µl). The opsonisation was performed at 37 °C for 30 min while shaking. Plates were then placed on ice and 2.5 * 10^5 human polymorphonuclear cells isolated from peripheral blood of healthy volunteers were added to each well (final volume 100 µl). Human PMNs were isolated by mixing 30 ml of heparinised blood with 30 ml of phosphate-buffered saline (pH 7.4), layered on Ficoll-Paque, and centrifuged for 20 min at 400*g. The lowest layer containing PMNs and erythrocytes was washed once in RPMI (Gibco BRL, Life Technologies LTD, Paisley, UK) containing 0,05% human serum albumin. The erythrocytes were lysed using ice-cold lysis buffer (155 mM NH_4Cl, 10 mM $KHCO_3$, 1 mM EDTA, pH 7.4). Phagocytosis was performed for 30 min at 37 °C while shaking. After washing twice with ice-cold HBSS, samples were resuspended in 200 µl of HBSS. The PMNs were fixed by adding 100 µl PBS-2% paraformaldehyde, and the samples were analysed in a FACScan flow cytometer (Becton Dickinson). Fluorescent PMNs observed after opsonisation with antiserum indicates both uptake and binding (referred to as phagocytosis) of FITC-labelled bacteria. The opsonophagocytic activity is defined as the reciprocal of the serum concentration at which 25 % of the human PMNs were fluorescent.

Immuno electron microscopy.

Immuno electron microscopy was performed according to the standard operational procedures of the national institute for biological standards and control, Potters bar, United Kingdom.

Purification, tryptic digest and mass spectrometric analysis of the proteins.

The protein gel spots of interest were excised from the gel. The gel fragments were sliced thinly and washed twice for 15 minutes in 5 % trichloro acetic acid

($C_2HCl_3O_2$; Merck, Darmstadt, Germany) and three times in distilled water. The gel fragments were equilibrated in sample buffer pH 6.8
(0.1 % SDS, 10 % glycerol, 50 mM DTT, 12 mM Tris-HCl, 0.01 % bromophenol-blue) for 1 hour at room temperature.

The proteins were concentrated by an agarose electrophoresis (1 % agarose type VIII, Sigma, St. Louis, USA) method as described by Rider et al. (Rider, M. H., M. Puype, J. van Damme, K. Gevaert, S. de Boeck, J. D'Alayer, H. H. Rasmussen, J. E. Celis, and J. Vanderkerckhove. 1995. An agarose-based gel-concentration system for microsequence and mass spectrometric characterization of proteins previously purified in polyacrylamide gels starting at low picomole levels. Eur. J. Biochem. **230**:258-265.) and Gevaert et al. (Gevaert, K., J. Verschelde, M. Puype, J. van Damme, M. Goethals, S. de Boeck, and J. Vanderkerckhove. 1996. Structural analysis and identification of gel-purified proteins in the femtomole range, using a novel computer program for peptide sequence assignment, by matrix-assisted laser desorption ionisation-reflection time-of-flight-mass spectrometry. Electrophoresis. **17**:918-924) on a Bio-Rad model 150-A gel electrophoresis cell (Bio-Rad laboratories, Richmond, USA) with Pasteur pipettes. After staining the agarose gel with carconcarboxylic acid (Sigma), the proteins were excised from the gel. The agarose fragments were washed with distilled water, and resuspended in 18 µl of digestion buffer pH 8.0 (50 mM NH_4HCO_3, 5 mM $CaCl_2$). The agarose was melted at 85 °C for 1 minute. After cooling down to 37 °C 0.05 µg/µl trypsin (trypsin modified sequencing grade, Promega, Madison, USA) was added to digest the proteins for at least 15 hours at 37 °C. Trypsin was inactivated by adding 1 µl of 10 % trifluoro acetic acid ($C_2HF_3O_2$; Merck).

The tryptic digests were analysed using a reversed phase micro-capillary column switching HPLC system (Meiring, H. D., B. M. Barroso, E. van der Heeft, G. J. ten Hove, and A. P. J. M. de Jong. 1999. Sheathless *Nano*flow HPLC-ESI/MS(n) in Proteome Research and MHC Bound Peptide Identification. In Proceedings of the 47th ASMS Conference on Mass Spectrometry and Allied Topics, Dallas, Texas.; van der Heeft, E., G. J. ten Hove, C. A. Herberts, H. D. Meiring, C. A. C. M. van Els, and A. P. J. M. de Jong. 1998. A microcapillary column switching system HPLC-electrospray ionisation MS system for the direct identification of peptides presented by major histocompatibility complex class I molecules. Anal. Chem. **70**:3742-3751.).

Peptide sequencing was performed on a LCQ quadropole ion trap mass spectrometer (Finnigan MAT, San Jose, CA, USA). Tandem mass spectrometric data were collected in data dependent scan mode for sequence information of single tryptic digest products. With Peptide Search (Mann, M., and M. Wilm. 1994. Error-tolerant identification of peptides in sequence databases by peptide sequence tags. Anal. Chem. **66**:4390-4399.), the deduced (partial) amino acid sequences were analysed for matching sequences in all possible translation products of the most current version of the unfinished pneumococcal genome released by The Institute for Genomic Research (TIGR; http://www.tigr.org/data/s_pneumoniae/) to identify the proteins. With the BLAST algorithm (Altschul, S. F., G. W. Miller, E. W. Myers, and D. J. Lipman. 1990. Basic local alignment search tool. J. Mol. Biol. **251**:403-410), putative pneumococcal proteins were analysed for similarity to sequences deposited in the November 1999 version of the non-redundant protein database at the National Center for Biotechnology Information (Washington D.C., USA).

Further proof of principle can be obtained by immunisation experiments in various animal models (mice, rats, rabbits) using purified Pmp, recombinant Pmp or derivatives ad fragments of Pmp.

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Claims

1. A vaccine or medical preparation comprising a protease maturation protein of *S. pneumoniae* and/or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof for the treatment of microbial infections.
2. The vaccine or medical preparation according to claim 1 for the treatment of *S. pneumoniae.*
3. The vaccine or medical preparation according to claim 1 or 2, further comprising a suitable adjuvant or carrier.
4. The vaccine or medical preparation according to anyone of the claims 1-3, wherein said protein comprises an amino acid sequence as shown in fig 1B.
5. The vaccine or medical according to anyone of the claims 1-4, wherein said protein is the protein maturation protein from *S. pneumoniae* Ft231 or EF3296.
6. The vaccine or medical preparation according to anyone of the claims 1-5, wherein said fragment comprises an anchoring fragment, an antigenic fragment or a functional equivalent thereof or a functional equivalent of a receptor binding site or an antibody binding site.
7. The vaccine or medical preparation according to anyone of the claims 1-6, wherein said protein or said fragment comprises a purified, partly purified, recombinant or synthetic protein or fragment thereof.
8. The vaccine or medical preparation according to anyone of the claims 1-7, wherein said fragment comprises at least 8 amino acids.
9. Method for the preparation of a vaccine against *S. pneumoniae* comprising the steps of:

   a. isolating a protease maturation protein of *S. pneumoniae* or a fragment thereof or a recombinant or synthetic protein or fragment thereof or homologous or functionally homologous protein or fragment thereof; and

   b. combining the protein or the fragment thereof obtained under (a) with a suitable carrier or adjuvant.
10. Method for obtaining an antibody against the protease maturation protein of *S. pneumoniae,* comprising the steps of isolating said protease maturation protein

or a fragment thereof, and raising antibodies against said protein or fragment thereof.

11. Antibody obtainable by the method according to claim 10.

12. Use of a protease maturation protein of *S. pneumoniae* or a fragment thereof for the preparation of a vaccine for the treatment or prophylaxis of a *S. pneumoniae* infection.

13. Use of a protease maturation protein of *S. pneumoniae* or a fragment thereof or a recombinant or synthetic protein or fragment thereof as a carrier.

14. Method of treatment of a *S. pneumoniae* infection comprising administering a vaccine according to claims 1-8.

15. Method for the vaccination of a mammal against an infection of *S. pneumoniae* comprising administering a suitable dose of a vaccine according to anyone of the claims 1-8.

16. Use of a nucleic acid sequence encoding for a protease maturation protein or a fragment thereof for obtaining a recombinant protease maturation protein or fragment thereof.

17. Cell containing a recombinant nucleic acid sequence or a vector encoding for protease maturation protein or a fragment thereof.

18. Recombinant protease maturation protein or fragment thereof obtainable through the expression of a gene sequence encoding for said protein in a suitable vector.

19. Use of protease maturation protein of *S. pneumoniae* and/or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof for the preparation of a medicament for the treatment of diseases connected with *S. pneumoniae* infections.

20. Use of protease maturation protein of *S. pneumoniae* and/or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof for eliciting opsonophagocytic activity and/or *in vivo* immunisation and/or *in vivo* immune protection against *S. pneumoniae.*

21. Method for the identification of proteins eliciting opsonophagocytic activity and/or *in vivo* immune protection comprising subjecting proteins to protein electrophoresis, preferably 2D, obtaining antisera against the surface associated

proteins, subjecting the isolated protein or fractions thereof to an immunocytometric assay and to an opsonophagocytic assay, in any order.

Figure 1.

Fig 1A.

AGTAACACTTATCTCAAAGGAGTAGACATGAAGAAAAAATTATTGGCAGGTG
CCATCACACTATTATCAGTAGCAACTTTAGCAGCTTGTTCGAAAGGGTCAGAAGGTG
CAGACCTTATCAGCATGAAAGGGGATGTCATTACAGAACATCAATTTTATGAGCAAG
TGAAAAGCAACCCTTCAGCCCAACAAGTCTTGTTAAATATGACCATCCAAAAAGTTT
TTGAAAAACAATATGGCTCAGAGCTTGATGATAAAGAGGTTGATGATACTATTGCCG
AAGAAAAAAAACAATATGGCGAAAACTACCAACGTGTCTTGTCACAAGCAGGTATGA
CTCTTGAAACACGTAAAGCTCAAATTCGTACAAGTAAATTAGTTGAGTTGGCAGTTA
AGAAGGTAGCAGAAGCTGAATTGACAGATGAAGCCTATAAGAAAGCCTTTGATGAGT
ACACTCCAGATGTAACGGCTCAAATCATCCGTCTTAATAATGAAGATAAGGCCAAAG
AAGTTCTCGAAAAAGCCAAGGCAGAAGGTGCTGATTTTGCTCAATTAGCCAAAGATA
ATTCAACTGATGAAAAAACAAAAGAAAATGGTGGAGAAATTACCTTTGATTCTGCTT
CAACAGAAGTACCTGAGCAAGTCAAAAAAGCCGCTTTCGCTTTAGATGTGGATGGTG
TTTCTGATGTGATTACAGCAACTGGCACACAAGCCTACAGTAGCCAATATTACATTG
TAAAACTCACTAAGAAAACAGAAAAATCATCTAATATTGATGACTACAAAGAAAAAT
TAAAAACTGTTATCTTGACTCAAAAACAAAATGATTCAACATTTGTTCAAAGCATTA
TCGGAAAAGAATTGCAAGCAGCCAATATCAAGGTTAAGGACCAAGCCTTCCAAAATA
TCTTTACCCAATATATCGGTGGTGGAGATTCAAGCTCAAGCAGTAGTACATCAAACG
AA

Fig 1B.

SNTYLKGVDMKKKLLAGAITLLSVATLAACSKGSEGADLISMKGDVITEHQF
YEQVKSNPSAQQVLLNMTIQKVFEKQYGSELDDKEVDDTIAEEKKQYGENYQRVLSQ
AGMTLETRKAQIRTSKLVELAVKKVAEAELTDEAYKKAFDEYTPDVTAQIIRLNNED
KAKEVLEKAKAEGADFAQLAKDNSTDEKTKENGGEITFDSASTEVPEQVKKAAFALD
VDGVSDVITATGTQAYSSQYYIVKLTKKTEKSSNIDDYKEKLKTVILTQKQNDSTFV
QSIIGKELQAANIKVKDQAFQNIFTQYIGGGDSSSSSSTSNE

Figure 2

mkkkmrlkvllastatallllsgcqsnqadqkvatysggkvtesnfykelkq
spttktmlanmliyralnhaygksvstktvndaydsykqqygenfdaflsqngfsrs
sfkeslrtnflsevalkklkkvsesqlkavwktyqpkvtvqhiltsdedtakqvisd
laagkdfatlaktdsidtatkdnggkisfesnnktldatfkdaayklkngdytqtpv
kvtngyevikminhpakgtftsskkaltasvyakwsrdssimqrvisqvlknqhvti
kdkdladaldsykkpattn

Figure 3

mkkkmrlkvllastatallllsgcqsnqtdqtvatysggkvtessfykelkq
spttktmlanmliyralnhaygksvstktvndaydsykqqygenfdaflsqngfsrs
sfkeslrtnflsevalkklkkvsesqlkaawktyqpkvtvqhiltsdedtakqvisd
laagkdfamlaktdsidtatkdnggkisfelnnktldatfkdaayklkngdytqtpv
kvtdgyevikminhpakgtftsskkaltasvyakwsrdssimqrvisqvlknqhvti
kdkdladaldsykklattn

Figure 4

mkkkmrlkvllastatallllsgcqsnqtdqtvatysggkvtesslykelkq
spttktmlanmliyralnhaygksvstktvndaydsykqqygenfdaflsqngfsrs
sfkeslrtnflsevalkklkkvsesqlkaawktyqpkvtvqhiltsdedtakqvisd
laagkdfamlaktdsidtatkdnggkisfelnnktldatfkdaayklkngdytqtpv
kvtdgyevikminhpakgtftsskkaltasvyakwsrdssimqrvisqvlknqhvti
kdkdladaldsykklattn

INTERNATIONAL SEARCH REPORT

International Application No
PCT/NL 00/00569

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/09 C07K14/315 C12N15/31 C07K16/12 A61P31/04
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, PAJ, MEDLINE, CHEM ABS Data, EMBASE, LIFESCIENCES SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 18930 A (HUMAN GENOME SCIENCES) 7 May 1998 (1998-05-07) cited in the application *page 55, see sequence SP021* page 114 -page 116	1-21
A	WO 97 37026 A (SMITHKLINE BEECHAM) 9 October 1997 (1997-10-09) cited in the application page 346 -page 348	1-21

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

° Special categories of cited documents :

°A° document defining the general state of the art which is not considered to be of particular relevance

°E° earlier document but published on or after the international filing date

°L° document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

°O° document referring to an oral disclosure, use, exhibition or other means

°P° document published prior to the international filing date but later than the priority date claimed

°T° later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

°X° document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

°Y° document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

°&° document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 January 2001	05/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL – 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

1

Form PCT/ISA/210 (second sheet) (July 1992)

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MCDANIEL L S ET AL: "COMPARISON OF THE PSPA SEQUENCE FROM STREPTOCOCCUS PNEUMONIAE EF5668 TO THE PREVIOUSLY IDENTIFIED PSPA SEQUENCE FROM STRAIN RX1 AND ABILITY OF PSPA FROM EF5668 TO ELICIT PROTECTION AGAINST PNEUMOCOCCI OF DIFFERENT CAPSULAR TYPES" INFECTION AND IMMUNITY,AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON,US, vol. 66, no. 10, October 1998 (1998-10), pages 4748-4754, XP000918186 ISSN: 0019-9567 the whole document	1-21
A	JANSEN W T M ET AL: "Use of highly encapsulated Streptococcus pneumoniae strains in a flow-cytometric assay for assessment of the phagocytic capacity of serotype-specific antibodies." CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, vol. 5, no. 5, 1998, pages 703-710, XP002158136 ISSN: 1071-412X the whole document	1-21
P,X	WO 00 06737 A (MICROBIAL TECHNICS LIMITED) 10 February 2000 (2000-02-10) cited in the application the whole document	1-21
P,X	OVERWEG K ET AL: "The putative proteinase maturation protein A of Streptococcus pneumoniae is a conserved surface protein with potential to elicit protective immune responses." INFECTION AND IMMUNITY, vol. 68, no. 7, July 2000 (2000-07), pages 4180-4188, XP002158137 ISSN: 0019-9567 the whole document	1-21

INTERNATIONAL SEARCH REPORT

Information on patent family members

International Application No
PCT/NL 00/00569

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9818930 A	07-05-1998	AU 5194598 A	22-05-1998
		AU 6909098 A	22-05-1998
		EP 0942983 A	22-09-1999
		EP 0941335 A	15-09-1999
		WO 9818931 A	07-05-1998
		US 6159469 A	12-12-2000
WO 9737026 A	09-10-1997	EP 0907738 A	14-04-1999
		JP 2000511769 T	12-09-2000
WO 0006737 A	10-02-2000	NONE	

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(81) Designated States *(national)*: AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, BZ, CA, CH, CN, CR, CU, CZ, DE, DK, DM, DZ, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW.

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Published:
— *With international search report.*
— *Before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments.*

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WO 01/12219 A1

(54) Title: PNEUMOCOCCAL VACCINES

(57) Abstract: The invention relates to the use of a protein or a fragment thereof of *S. pneumoniae*, its use for the preparation of a vaccine for the preventive treatment of a *S. pneumoniae* infection, compositions comprising protease maturation protein of *S. pneumoniae* infection, or a fragment there f, vaccines comprising said protein or fragment thereof, use of a nucleic acid sequence encoding for said protein or fragment thereof, vectors wherein the nucleic acid sequence is brought to expression and to recombinant protease maturation protein r a fragment thereof or (functi nal) homologues thereof and to a method for the determination of proteins with opsonophagocytic activity and/or *in vivo* immunisation and/or *in vivo* immune protection.

PATENT COOPERATION TREATY

ONTVANGEN

12 NOV 2001

AMERSFOORT

From the
INTERNATIONAL PRELIMINARY EXAMINING AUTHORITY

To:

PRINS, Ir A.W.
VEREENIGDE
Nieuwe Parklaan 97
2587 BN Den Haag
PAYS-BAS

NOTIFICATION OF TRANSMITTAL OF THE INTERNATIONAL PRELIMINARY EXAMINATION REPORT
(PCT Rule 71.1)

Date of mailing *(day/month/year)*	23.10.2001
Applicant's or agent's file reference	P50337PC00
	IMPORTANT NOTIFICATION
International application No.	PCT/NL00/00569
International filing date *(day/month/year)*	14/08/2000
Priority date *(day/month/year)*	13/08/1999
Applicant	ERASMUS UNIVERSITEIT ROTTERDAM et al.

1. The applicant is hereby notified that this International Preliminary Examining Authority transmits herewith the international preliminary examination report and its annexes, if any, established on the international application.

2. A copy of the report and its annexes, if any, is being transmitted to the International Bureau for communication to all the elected Offices.

3. Where required by any of the elected Offices, the International Bureau will prepare an English translation of the report (but not of any annexes) and will transmit such translation to those Offices.

4. **REMINDER**

The applicant must enter the national phase before each elected Office by performing certain acts (filing translations and paying national fees) within 30 months from the priority date (or later in some Offices) (Article 39(1)) (see also the reminder sent by the international Bureau with Form PCT/IB/301).

Where a translation of the international application must be furnished to an elected Office, that translation must contain a translation of any annexes to the international preliminary examination report. It is the applicant's responsibility to prepare and furnish such translation directly to each elected Office concerned.

For further details on the applicable time limits and requirements of the elected Offices, see Volume II of the PCT Applicant's Guide.

Name and mailing address of the IPEA/	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Digiusto, M Tel.+49 89 2399-8162

Form PCT/IPEA/416 (July 1992)

PATENT COOPERATION TREATY

PCT

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

(PCT Article 36 and Rule 70)

Applicant's or agent's file reference	FOR FURTHER ACTION	See Notification of Transmittal of International Preliminary Examination Report (Form PCT/IPEA/416)
P50337PC00		
International application No.	International filing date *(day/month/year)*	Priority date *(day/month/year)*
PCT/NL00/00569	14/08/2000	13/08/1999
International Patent Classification (IPC) or national classification and IPC		
A61K39/09		
Applicant		
ERASMUS UNIVERSITEIT ROTTERDAM et al.		

1. This international preliminary examination report has been prepared by this International Preliminary Examining Authority and is transmitted to the applicant according to Article 36.

2. This REPORT consists of a total of 9 sheets, including this cover sheet.

   ☒ This report is also accompanied by ANNEXES, i.e. sheets of the description, claims and/or drawings which have been amended and are the basis for this report and/or sheets containing rectifications made before this Authority (see Rule 70.16 and Section 607 of the Administrative Instructions under the PCT).

   These annexes consist of a total of 3 sheets.

3. This report contains indications relating to the following items:

   - I ☒ Basis of the report
   - II ☒ Priority
   - III ☒ Non-establishment of opinion with regard to novelty, inventive step and industrial applicability
   - IV ☐ Lack of unity of invention
   - V ☒ Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations suporting such statement
   - VI ☐ Certain documents cited
   - VII ☐ Certain defects in the international application
   - VIII ☒ Certain observations on the international application

Date of submission of the demand	Date of completion of this report
12/03/2001	23.10.2001
Name and mailing address of the international preliminary examining authority:	Authorized officer
European Patent Office D-80298 Munich Tel. +49 89 2399 - 0 Tx: 523656 epmu d Fax: +49 89 2399 - 4465	Leber, T Telephone No. +49 89 2399 7195

Form PCT/IPEA/409 (cover sheet) (January 1994)

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL00/00569

I. Basis of the report

1. With regard to the **elements** of the international application *(Replacement sheets which have been furnished to the receiving Office in response to an invitation under Article 14 are referred to in this report as "originally filed" and are not annexed to this report since they do not contain amendments (Rules 70.16 and 70.17))*:
Description, pages:

1-21 as originally filed

Claims, No.:

1-19 as received on 21/09/2001 with letter of 20/09/2001

Drawings, sheets:

1/2,2/2 as originally filed

Sequence listing part of the description, pages:

1-8, filed with the letter of 03.11.2000

2. With regard to the **language**, all the elements marked above were available or furnished to this Authority in the language in which the international application was filed, unless otherwise indicated under this item.

These elements were available or furnished to this Authority in the following language: , which is:

- ☐ the language of a translation furnished for the purposes of the international search (under Rule 23.1(b)).
- ☐ the language of publication of the international application (under Rule 48.3(b)).
- ☐ the language of a translation furnished for the purposes of international preliminary examination (under Rule 55.2 and/or 55.3).

3. With regard to any **nucleotide and/or amino acid sequence** disclosed in the international application, the international preliminary examination was carried out on the basis of the sequence listing:

- ☐ contained in the international application in written form.
- ☐ filed together with the international application in computer readable form.
- ☒ furnished subsequently to this Authority in written form.
- ☒ furnished subsequently to this Authority in computer readable form.
- ☒ The statement that the subsequently furnished written sequence listing does not go beyond the disclosure in the international application as filed has been furnished.
- ☒ The statement that the information recorded in computer readable form is identical to the written sequence listing has been furnished.

4. The amendments have resulted in the cancellation of:

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL00/00569

- ☐ the description, pages:
- ☐ the claims, Nos.:
- ☐ the drawings, sheets:

5. ☐ This report has been established as if (some of) the amendments had not been made, since they have been considered to go beyond the disclosure as filed (Rule 70.2(c)):

   (Any replacement sheet containing such amendments must be referred to under item 1 and annexed to this report.)

6. Additional observations, if necessary:

II. Priority

1. ☐ This report has been established as if no priority had been claimed due to the failure to furnish within the prescribed time limit the requested:

   ☐ copy of the earlier application whose priority has been claimed.

   ☐ translation of the earlier application whose priority has been claimed.

2. ☐ This report has been established as if no priority had been claimed due to the fact that the priority claim has been found invalid.

   Thus for the purposes of this report, the international filing date indicated above is considered to be the relevant date.

3. Additional observations, if necessary:
   see separate sheet

III. Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. The questions whether the claimed invention appears to be novel, to involve an inventive step (to be non-obvious), or to be industrially applicable have not been examined in respect of:

   ☐ the entire international application.

   ☒ claims Nos. 1-19(partly);13,14,19(IA).

because:

☒ the said international application, or the said claims Nos. 13,14,19(IA) relate to the following subject matter which does not require an international preliminary examination (*specify*):
see separate sheet

☐ the description, claims or drawings (*indicate particular elements below*) or said claims Nos. are so unclear that no meaningful opinion could be formed (*specify*):

INTERNATIONAL PRELIMINARY EXAMINATION REPORT

International application No. PCT/NL00/00569

☐ the claims, or said claims Nos. are so inadequately supported by the description that no meaningful opinion could be formed.

☒ no international search report has been established for the said claims Nos. 1-19(partly).

2. A meaningful international preliminary examination cannot be carried out due to the failure of the nucleotide and/or amino acid sequence listing to comply with the standard provided for in Annex C of the Administrative Instructions:

☐ the written form has not been furnished or does not comply with the standard.

☐ the computer readable form has not been furnished or does not comply with the standard.

V. Reasoned statement under Article 35(2) with regard to novelty, inventive step or industrial applicability; citations and explanations supporting such statement

1. Statement

Novelty (N)	Yes:	Claims	
	No:	Claims	1-11,13-17,19(partly)
Inventive step (IS)	Yes:	Claims	
	No:	Claims	12,18(partly)
Industrial applicability (IA)	Yes:	Claims	1-12,15-18(partly)
	No:	Claims	

2. Citations and explanations
see separate sheet

VIII. Certain observations on the international application

The following observations on the clarity of the claims, description, and drawings or on the question whether the claims are fully supported by the description, are made:
se separate sheet

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/NL00/00569

Re Item I

Basis of the opinion

1. Sequence listing pages 1-8 filed with the letter of 03.11.2000 do not form part of the application (Rule 13ter.1(f) PCT).

Re Item II

Priority

1. Priority of the present patent application was checked and found partly valid. The following sections of the description are not part of the priority document: page 2, lines 7-22; page 9, lines 20-27; page 10, line 30 - page 11, line 21; page 15, line 29 - page 17, line 18.

Re Item III

Non-establishment of opinion with regard to novelty, inventive step and industrial applicability

1. Claims 13, 14 and 19 relate to subject-matter considered by this Authority to be covered by the provisions of Rule 67.1(iv) PCT. Consequently, no opinion will be formulated with respect to the industrial applicability of the subject-matter of these claims (Article 34(4)(a)(i) PCT).

2. The sequence disclosed in Fig. 1b of the present application differs from the sequence provided in the sequence listing (Seq ID NO:2) by the first 9 amino acids. As the International Search Report is based on Seq ID NO:2, claims 1-19 have only partly been searched and examination will thus be restricted to searched parts (Rule 66.1(e) PCT).

Re Item V

Reasoned statem nt und r Rul 66.2(a)(ii) with regard to novelty, inventive step or industrial applicability; citati ns and xplanation supp rting such statement

INTERNATIONAL PRELIMINARY EXAMINATION REPORT - SEPARATE SHEET

International application No. PCT/NL00/00569

1. Basis for the assessment of novelty, inventive step and industrial applicability

1.1 Reference is made to the following documents:

D1: WO 98 18930 A (HUMAN GENOME SCIENCES) 7 May 1998 (1998-05-07) cited in the application

D2: WO 00 06737 A (MICROBIAL TECHNICS LIMITED) 10 February 2000 (2000-02-10) cited in the application

1.2 The amendments filed with the letter of 20.09.2001 fulfill the requirements of Art 34(2)(b) PCT.

2. Novelty

2.1 Document D1 discloses antigens and vaccines to prevent or attenuate infections caused by bacteria of the Streptococcus genus and S. pneumoniae in particular (D1, Abstract; page 115, claims 16 and 17). The vaccine encompasses a polypeptide or a fragment thereof contained in table 1 of D1 (D1, page 114, claim 16). Table 1 of D1 discloses SEQ ID 34, which is over a stretch of 141 amino acids (SKG...TEV) identical to that referred to in Fig. 1b of the present application. The vaccine may be prepared with a carrier and is suitable to elicit protective antibodies in the vaccinated animal (D1, page 114, claim 16). The peptides can be produced recombinantly (D1, page 3, line 36 - page 4, line 5) and encompass at least nine amino acids (D1, page 25, line 1). Moreover, the peptides may be used for antibody production (D1, claims 11 and 15). In light of the information provided in D1 it appears that D1 is fully enabling for the skilled person. Therefore claims 1-3, 8, 9, 11, 13-17, 19 lack novelty (Art 33(2) PCT).
This judgement is not altered by the fact that the name of the protein referred to in claim 1 ("protease maturation protein") is not disclosed in D1 as the name does not represent a distinguishing technical feature. Moreover, it is of no relevance that D1 does not disclose a opsonophagocytic response as this represents an intrinsic feature of the product referred to in claim 1, which is, as outlined above, not novel over D1. Thus, the product used in D1 has the same intrinsic feature of causing an opsonophagocytic response upon vaccination (see also ITEM V-3.1

below).

2.2 Claim 4 defines two strains from which the protease maturation protein can be derived. These strains do not provide a relevant feature for the assessment of novelty as the source of a product does not render the product novel. Thus claim 4 and also claims 5-7 lack novelty (Art 33(2) PCT).

2.3 Claim 10 represents product by process claim. Such claims are only allowable if each possible product is novel and inventive (Art 33(2) and Art 33(3) PCT). D1 discloses a segment of the polypeptide and its use for antibody production (see 2.1 above). In other words, antibodies are raised against the same target in D1 and in the present application. Therefore, claim 10 lacks novelty (Art 33(2) PCT).

3. Inventive step

3.1 Claim 12 differs from the closest prior art document D1 by the use of the protease maturation protein or a fragment thereof as a carrier. The term "carrier" is commonly understood as being a macromolecule suitable for enhancing the immunogenicity of the polypeptides. Examples are keyhole limpet hemacyanin (KLH), tetanus toxoid, pertussis toxin, bovine serum albumin and ovalbumin (e.g. D1, page 39, line 4-14). Thus, the function of the carrier appears to be to improve the epitope of the small peptide for the immune response of the challenged animal. It is therefore obvious for the person skilled in the art that in principal any protein could be used as a carrier. Moreover opsonisation represents a biological activity associated with mononuclear phagocytes and granulocytes which have the ability to ingest particulate matter. Both cell type mentioned above express cell surface receptors for various types of antibodies so that each matter which is bound to an antibody may be ingested by opsonisation once it is bound to an antibody. Therefore, activity of causing opsonophagocytosis appears not to be a no surprising effect of the carrier referred to in claim 12 but associated with any matter that may cause antibody production, e.g. the carriers mentioned above. In conclusion, claim 12 lacks an inventive step (Art 33(3) PCT).

3.2 Claim 18 refers to the use of the protease maturation prot in or a fragment thereof for the preparation of a medicament for the treatment of diseases connected with

S. pneumonia infections. Claim 18 differs from the closest prior art documents D1 and D2 in that the medicament is for the treatment of diseases **connected** with S. pneumonia infections and not for the S. pneumonia infection as such.
The technical problem is thus an improved spectrum of applicability of the said medicament. An inventive step cannot be acknowledged (Art 33(3) PCT) for the solution of said problem as it is obvious for a person skilled in the art that a medicament which fights an infection is also of use for diseases which result from the said infection. As discussed above (see ITEM V-2.1) D1 appears to be enabling and is thus relevant prior art.

4. Industrial applicability

4.1 The subject-matter disclosed in the claims 8-10, 12, 15-17 of the present application appears to be industrially applicable (Art 33(4) PCT).

4.2 For the assessment of the present claims 1-7, 11, 13, 14, 18 and 19 on the question whether they are industrially applicable, no unified criteria exist in the PCT Contracting States. The patentability can also be dependent upon the formulation of the claims. The EPO, for example, does not recognize as industrially applicable the subject-matter of claims to the use of a compound in medical treatment, but may allow, however, claims to a known compound for first use in medical treatment and the use of such a compound for the manufacture of a medicament for a new medical treatment.

Re Item VIII
Certain observations on the international application

1. Claim 1 lacks clarity (Art 6 PCT). The name "protease maturation protein" is insufficient to define the protein concerned. Further, the said protein "comprises" the amino acid sequence as shown in Fig. 1B. It is thus not clear whether or not the said amino acid sequence defines already a protease maturation protein (Art 6 PCT). Moreover, th t rms "fragment", "homologous", "functional homologous", "functional fragment" lack clarity (Art 6 PCT) as there is no clear definition how, for example, a fragm nt has to look like to be still a "prot as maturation" and to still

have the relevant function. No clarification can be derived from the description for these terms. The term "fragment" is on the one side defined functionally (page 7, line 17) and on the other side in terms of the peptide length (page 8, lines 6-7) without linking these definitions so as to render it clear whether or not, for example, the required biological activity is present. Moreover, the function of Pmp appears to be insufficiently disclosed as it is only based on sequence homology analysis (page 6, lines 24-29).
At least some of the said objections apply also to claims 5, 6, 8, 9, 11, 12, 15-19.

2. Claims must not in respect of technical features rely on references to drawings (Rule 6.2a PCT). Amino acid sequences may be defined with sequence identification numbers. This objection applies to claims 1, 8, 9, 11, 12, 15-19.

3. The term "suitable" in claims 3 and 14 lacks clarity as no definition is given which permits the skilled person to distinguish between suitable and unsuitable carriers (Art 6 PCT).

4. The terms "anchoring fragment", "antigenic fragment or functional equivalent thereof" and "functional equivalent of a receptor binding site or a antibody binding site" in claim 6 lack clarity (Art 6 PCT).

5. Considering the nature of the invention, it appears that the number of 14 independent claims is excessive leading to a lack of conciseness (Guidelines, Section IV, III-5).

6. Claim 18 refers to the use of the protease maturation protein or a fragment thereof for the preparation of a medicament for the treatment of diseased **connected** with S. pneumonia infections. This feature appears not to be supported by the description (Art 6 PCT).

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Our letter of 20 September 2001

Amended claims

1. A vaccine or medical preparation comprising a protease maturation protein of *S. pneumoniae* comprising an amino acid sequence as shown in fig 1B, and/or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof for the treatment of microbial infections.

2. The vaccine or medical preparation according to claim 1 for the treatment of *S. pneumoniae*.

3. The vaccine or medical preparation according to claim 1 or 2, further comprising a suitable adjuvant or carrier.

4 The vaccine or medical preparation according to anyone of the claims 1-3 wherein said protein is the protein maturation protein from *S. pneumoniae* Ft231 or EF3296.

5. The vaccine or medical preparation according to anyone of the claims 1-4 wherein said fragment comprises an anchoring fragment, an antigenic fragment or a functional equivalent thereof or a functional equivalent of a receptor binding site or an antibody binding site.

6 The vaccine or medical preparation according to anyone of the claims 1-5 wherein said protein or said fragment comprises a purified, recombinant or synthetic protein or fragment thereof.

7. The vaccine or medical preparation according to anyone of the claims 1-6 wherein said fragment comprises at least 8 amino acids.

8. Method for the preparation of a vaccine against *S. pneumoniae* comprising the steps of:

a. isolating a protease maturation protein of *S. pneumoniae* comprising an amino acid sequence as shown in fig 1B, or a fragment thereof or a recombinant or synthetic protein or fragment thereof or homologous or functionally homologous protein or fragment thereof; and

b. combining the protein or the fragment thereof obtained under (a) with a suitable carrier or adjuvant.

9. Method for obtaining an antibody against the protease maturation protein of *S. pneumoniae*, the method comprising the steps of isolating protease maturation protein comprising an amino acid sequence as shown in fig 1B or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof, and raising antibodies against said protein or fragment thereof.

10. Antibody comprising opsonophagocytic activity obtainable by the method according to claim 9.

11. Use of a protease maturation protein of *S. pneumoniae* comprising an amino acid sequence as shown in fig 1B, or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof, for the preparation of a vaccine for the treatment or prophylaxis of a *S. pneumoniae* infection.

12. Use of a protease maturation protein of *S. pneumoniae* comprising an amino acid sequence as shown in fig 1B, or a fragment thereof or a recombinant or synthetic protein or fragment thereof as a carrier.

13. Method of treatment of a *S. pneumoniae* infection comprising administering a vaccine according to claims 1-7.

14. Method for the vaccination of a mammal against an infection of *S. pneumoniae* comprising administering a suitable dose of a vaccine according to anyone of the claims 1-7.

15. Use of a nucleic acid sequence coding for a protease maturation protein comprising an amino acid sequence as shown in fig 1B, or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof, for obtaining a recombinant protease maturation protein or fragment thereof.

16. Cell containing a recombinant nucleic acid sequence or a vector encoding for protease maturation protein comprising an amino acid sequence as shown in fig 1B, or a fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof.

17. Recombinant protease maturation protein comprising an amino acid sequence as shown in fig 1B, or fragment thereof and/or a homologous and/or a functionally homologous protein or protein fragment thereof, obtainable through the expression of a gene sequence encoding for said protein in a suitable vector.

18. Use of protease maturation protein of *S. pneumoniae* comprising an amino acid sequence as shown in fig 1B, and/or a fragment thereof and/or a homologous and/or a

AMENDED SHEET

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PATENT COOPERATION TREATY

From the INTERNATIONAL BUREAU

PCT

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

(PCT Rule 47.1(c), first sentence)

To:
PRINS, A., W.
Vereenigde
Nieuwe Parklaan 97
NL-2587 BN The Hague
PAYS-BAS

Kopie in/naar

2 MAART 2001

Date of mailing (day/month/year)
22 February 2001 (22.02.01)

Applicant's or agent's file reference
MAPP50337PC00

IMPORTANT NOTICE

International application No.	International filing date (day/month/year)	Priority date (day/month/year)
PCT/NL00/00569	14 August 2000 (14.08.00)	13 August 1999 (13.08.99)

Applicant
ERASMUS UNIVERSITEIT ROTTERDAM et al

1. Notice is hereby given that the International Bureau has communicated, as provided in Article 20, the international application to the following designated Offices on the date indicated above as the date of mailing of this Notice:
   AU,KP,KR,US

   In accordance with Rule 47.1(c), third sentence, those Offices will accept the present Notice as conclusive evidence that the communication of the international application has duly taken place on the date of mailing indicated above and no copy of the international application is required to be furnished by the applicant to the designated Office(s).

2. The following designated Offices have waived the requirement for such a communication at this time:
   AE,AG,AL,AM,AP,AT,AZ,BA,BB,BG,BR,BY,BZ,CA,CH,CN,CR,CU,CZ,DE,DK,DM,DZ,EA,EE,EP,ES,FI,GB,GD,GE,GH,GM,HR,HU,ID,IL,IN,IS,JP,KE,KG,KZ,LC,LK,LR,LS,LT,LU,LV,MA,MD,MG,MK,MN,MW,MX,MZ,NO,NZ,OA,PL,PT,RO,RU,SD,SE,SG,SI,SK,SL,TJ,TM,TR,TT,TZ,UA,UG,UZ,VN,YU,
   The communication will be made to those Offices only upon their request. Furthermore, those Offices do not require the applicant to furnish a copy of the international application (Rule 48.1(a-bis)).

3. Enclosed with this Notice is a copy of the international application as published by the International Bureau on
   22 February 2001 (22.02.01) under No. WO 01/12219

REMINDER REGARDING CHAPTER II (Article 31(2)(a) and Rule 54.2)

If the applicant wishes to postpone entry into the national phase until 30 months (or later in some Offices) from the priority date, a **demand for international preliminary examination** must be filed with the competent International Preliminary Examining Authority before the expiration of 19 months from the priority date.

It is the applicant's sole responsibility to monitor the 18-month time limit.

Note that only an applicant who is a national or resident of a PCT Contracting State which is bound by Chapter II has the right to file a demand for international preliminary examination.

REMINDER REGARDING ENTRY INTO THE NATIONAL PHASE (Article 22 or 39(1))

If the applicant wishes to proceed with the international application in the **national phase**, he must, within 20 months or 30 months, or later in some Offices, perform the acts referred to therein before each designated or elected Office.

For further important information on the time limits and acts to be performed for entering the national phase, see the Annex to Form PCT/IB/301 (Notification of Receipt of Record Copy) and Volume II of the PCT Applicant's Guide.

The International Bureau of WIPO 34, chemin des Colombettes 1211 Geneva 20, Switzerland	Authorized officer J. Zahra
Facsimile No. (41-22) 740.14.35	Telephone No. (41-22) 338.83.38

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Continuation of Form PCT/IB/308

NOTICE INFORMING THE APPLICANT OF THE COMMUNICATION OF THE INTERNATIONAL APPLICATION TO THE DESIGNATED OFFICES

Date of mailing (day/m nth/year) 22 February 2001 (22.02.01)	IMPORTANT NOTICE
Applicant's or agent's file reference P50337PC00	International application No. PCT/NL00/00569

The applicant is hereby notified that, at the time of establishment of this Notice, the time limit under Rule 46.1 for making amendments under Article 19 has not yet expired and the International Bureau had received neither such amendments nor a declaration that the applicant does not wish to make amendments.

INTERNATIONAL SEARCH REPORT

Interr. nal Application No
PCT/NL 00/00569

A. CLASSIFICATION OF SUBJECT MATTER
IPC 7 A61K39/09 C07K14/315 C12N15/31 C07K16/12 A61P31/04
G01N33/68

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 7 C07K C12N A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, WPI Data, BIOSIS, PAJ, MEDLINE, CHEM ABS Data, EMBASE, LIFESCIENCES SCISEARCH

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 98 18930 A (HUMAN GENOME SCIENCES) 7 May 1998 (1998-05-07) cited in the application *page 55, see sequence SP021* page 114 -page 116	1-21
A	WO 97 37026 A (SMITHKLINE BEECHAM) 9 October 1997 (1997-10-09) cited in the application page 346 -page 348	1-21

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents:

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 January 2001	05/02/2001

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax: (+31-70) 340-3016	Moreau, J

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

Intern .ial Application No
PCT/NL 00/00569

C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MCDANIEL L S ET AL: "COMPARISON OF THE PSPA SEQUENCE FROM STREPTOCOCCUS PNEUMONIAE EF5668 TO THE PREVIOUSLY IDENTIFIED PSPA SEQUENCE FROM STRAIN RX1 AND ABILITY OF PSPA FROM EF5668 TO ELICIT PROTECTION AGAINST PNEUMOCOCCI OF DIFFERENT CAPSULAR TYPES" INFECTION AND IMMUNITY,AMERICAN SOCIETY FOR MICROBIOLOGY. WASHINGTON,US, vol. 66, no. 10, October 1998 (1998-10), pages 4748-4754, XP000918186 ISSN: 0019-9567 the whole document	1-21
A	JANSEN W T M ET AL: "Use of highly encapsulated Streptococcus pneumoniae strains in a flow-cytometric assay for assessment of the phagocytic capacity of serotype-specific antibodies." CLINICAL AND DIAGNOSTIC LABORATORY IMMUNOLOGY, vol. 5, no. 5, 1998, pages 703-710, XP002158136 ISSN: 1071-412X the whole document	1-21
P,X	WO 00 06737 A (MICROBIAL TECHNICS LIMITED) 10 February 2000 (2000-02-10) cited in the application the whole document	1-21
P,X	OVERWEG K ET AL: "The putative proteinase maturation protein A of Streptococcus pneumoniae is a conserved surface protein with potential to elicit protective immune responses." INFECTION AND IMMUNITY, vol. 68, no. 7, July 2000 (2000-07), pages 4180-4188, XP002158137 ISSN: 0019-9567 the whole document	1-21

1

Form PCT/ISA/210 (continuation of second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

information on patent family members

International Application No
PCT/NL 00/00569

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
WO 9818930 A	07-05-1998	AU 5194598 A	22-05-1998
		AU 6909098 A	22-05-1998
		EP 0942983 A	22-09-1999
		EP 0941335 A	15-09-1999
		WO 9818931 A	07-05-1998
		US 6159469 A	12-12-2000
WO 9737026 A	09-10-1997	EP 0907738 A	14-04-1999
		JP 2000511769 T	12-09-2000
WO 0006737 A	10-02-2000	NONE	